Flow cytometric testing of susceptibilities of *Mycobacterium avium* to amikacin, ciprofloxacin, clarithromycin and rifabutin in 24 hours

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Objective To develop a biologically safe flow cytometric susceptibility test that depends on detection and enumeration of actively growing *Mycobacterium avium* organisms in drug-free and antimycobacterial agent-containing medium.

Methods Prior to analysis by flow cytometry, all *M. avium* susceptibility test samples were inactivated by exposure to paraformaldehyde. The susceptibilities of 20 clinical isolates of *M. avium* to amikacin, ciprofloxacin, clarithromycin, and rifabutin were tested by the flow cytometric and BACTEC methods.

Results Agreement was 97% between the results of the two methods. The results of flow cytometric susceptibility tests were available 24 h after inoculation of drug-containing medium, while the BACTEC method required 4–8 days to complete.

Conclusions The flow cytometric assay is safe, simple and reproducible.

Keywords Mycobacteria, susceptibility testing, flow cytometry

Accepted 23 March 1999

Clin Microbiol Infect 2000: 6: 366–373

INTRODUCTION

In the last two decades, the *Mycobacterium avium* complex (MAC), including *M. avium* and *M. intracellulare*, has become the most common mycobacterium recovered from patient specimens in the developed world [1–3]. The increasing incidence of MAC disease is largely due to the epidemic of acquired immunodeficiency syndrome (AIDS) [3–7]. MAC has been associated with approximately 90% of patients with AIDS [8]. Most patients with AIDS or symptomatic MAC infection develop disseminated multi-organ disease accompanied by continuous high-grade mycobacteremia [4–7,9–13]. In addition, infection with MAC is a significant problem in other groups of immunocompromised individuals, including those with cancer, chronic obstructive pulmonary disease, cystic fibrosis and diabetes mellitus [1,3,7]. Recently, reports have also suggested that MAC pulmonary disease can occur in individuals without predisposing conditions [5,14].

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Currently, a standardized method for susceptibility testing of MAC does not exist [3,13,15–18]. The broth method has been reported to be more reliable than the agar medium [3,9,15,16]; however, most laboratories prefer to use the BACTEC method, which requires 4–8 days of incubation before results are available [3,9,15,16,18]. Unfortunately, susceptibility testing for MAC continues to be problematic [1,2,5,9,18,19]. In fact, susceptibility testing of initial MAC isolates has been suggested to be inappropriate and unnecessary [3,9,17,20]. Despite these reservations, antimycobacterial susceptibility testing of MAC continues to be performed. Because therapy may be modified based on susceptibility results, it is not surprising that considerable efforts have been made to develop rapid and accurate tests for susceptibility testing of MAC [1,2,18,21,22].

Recently, we showed that accurate susceptibility testing of *M. tuberculosis* could be accomplished rapidly by enumeration of mycobacterial cells with a flow cytometer [19]. Results of tests were available 72 h after *M. tuberculosis* organisms were incubated with antimycobacterial agents. In this report, we demonstrate that enumeration of mycobacterial cells by flow cytometry can be used to detect the susceptibility or resistance of clinical isolates of *M. avium* to amikacin, ciprofloxacin, clar-

ithromycin and rifabutin 24 h after initiation of the testing procedure.

MATERIALS AND METHODS

Antimycobacterial agents

Amikacin, ciprofloxacin, clarithromycin and rifabutin were obtained from US Pharmacopeia (Rockville, MD, USA). For flow cytometry, stock solutions of amikacin and ciprofloxacin were prepared at 1000 mg/L with distilled water and sterilized by filtration with a 0.2-μm filter (Nalgene Labware Division, Rochester, NY, USA). Clarithromycin and rifabutin were also prepared at 1000 mg/L, but first dissolved in 0.4 mL of methanol (EM Science Industries, Inc., Gibbstown, NJ, USA). The pH of the stock solution of clarithromycin was adjusted to 6.5 with 0.1 M phosphate buffer. Clarithromycin and rifabutin were then incubated at 37 °C for 24 h for self-sterilization. Additional stock solutions were prepared for the radiometric broth method. These stock solutions were prepared similarly, except that amikacin was prepared at 360 mg/L and 80 mg/L, ciprofloxacin at 160 mg/L and 40 mg/L, clarithromycin at 1280 mg/L and 80 mg/L, and rifabutin at 80 mg/L and 4.8 mg/L. Aliquots of 1.0 mL of the sterilized antimycobacterial agents were frozen and stored at −70 °C until use.

Mycobacteria and preparation

Twenty clinical isolates of *M. avium* that varied in their resistance to antimycobacterial agents were obtained from the Wisconsin State Laboratory of Hygiene (WSLH, Madison, WI, USA). *M. avium* isolate 141 was obtained from the National Jewish Medical and Research Center (Denver, CO, USA). Initially, each isolate was grown in 7H9 broth (Difco, Detroit, MI, USA) at 37 °C with 7–8% CO_2 for approximately 5 days until the turbidity of the suspensions was equivalent to a McFarland 0.5 standard (1.0×10^8 CFU/mL). Each suspension was then dispensed in 1.0-mL aliquots into 1.5-mL screw-cap tubes (Sarstedt, Newton, NC, USA) and stored at −70 °C. When needed, a frozen suspension of each isolate of *M. avium* was thawed and an aliquot of 50 μL was used to inoculate 20 mL of fresh 7H9 broth in sterile 50-mL polypropylene screw-cap tubes (Sarstedt). Cultures were incubated for 72 h at 37 °C in the presence of 7–8% CO_2. The cultures were then used for susceptibility testing when the turbidity was equivalent to a McFarland 0.5 standard. Only log-phase cultures of *M. avium* were used for susceptibility testing.

Flow cytometric susceptibility testing

Aliquots of 0.5 mL of each actively growing *M. avium* isolate were transferred to 1.5-mL polypropylene screw-cap microtubes (Sarstedt). The tubes were then inoculated with 0.5 mL of: 64, 32, 16, 8, 4, 2, 1, 0.2, 0.02 or 0.002 mg of amikacin/L; 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.1, 0.02 or 0.002 mg of ciprofloxacin/L; 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.1, 0.02 or 0.002 mg of clarithromycin/L; and 16, 8, 4, 2, 1, 0.5, 0.24, 0.1, 0.02 or 0.002 mg of rifabutin/L to produce final concentrations of half of these amounts. Drug-free controls of *M. avium* were also prepared by inoculating them with 0.5 mL of 7H9 broth (Difco). Thereafter, the suspensions were incubated for 24 h at 37 °C in an environment of 7–8% CO_2. After incubation, 0.2 mL of each assay suspension was removed and placed in a sterile 1.5-mL screw-cap microtube containing 57 μL of 8% paraformaldehyde (pH 7.4) and 0.2 mL of sterile phosphate-buffered saline (final paraformaldehyde concentration was 1%). Samples were mixed and held at room temperature for 45 min before being analyzed with a Bryte HS flow cytometer with WinBryte software (Bio-Rad Laboratories, Hercules, CA, USA). Samples fixed with paraformaldehyde were periodically tested for viable cells by plating 0.01 mL of the suspension on 7H10 agar medium (Difco). Subsequently, the plates were incubated at 37 °C for 14 days. No viable *M. avium* cells were detected. After treatment with paraformaldehyde, *M. avium* cells were detected and differentiated from non-*M. avium* particles in 7H9 medium by flow cytometry using forward and side-angle light scatter signals. Electronic noise and background particles in the 7H9 medium were excluded from analysis by adjusting the threshold monitor listed on the WinBryte software program. Forward and side-angle light scatter were then used to analyze *M. avium* cells that were incubated with or without antimycobacterial agents. For each sample acquired, the flow cytometer provided a histogram profile showing the number of *M. avium* organisms in each of 2048 logarithmic channels of increasing light scatter. Five thousand events were acquired for each sample at a flow rate of 2 μL/min. In addition, 2.5-μm polystyrene beads (Molecular Probes, Eugene, OR, USA) were used daily for calibration of the instrument.

Flow cytometric susceptibility index

The susceptibility index was obtained by dividing the number of *M. avium* cells (events) detected in the drug-containing sample by the number of *M. avium* cells in the drug-free control. The susceptibility index of the control is 1. An isolate was considered susceptible if a susceptibility index of 0.65, 0.60, 0.55 or 0.55 or less was obtained after exposure to amikacin, ciprofloxacin, clarithromycin and rifabutin, respectively. The number of *M. avium* organisms/mL was obtained as part of the flow cytometric statistical analysis and was dependent upon establishment of gates to eliminate electronic noise and background particles from the medium.

Radiometric broth method (BACTEC)

Initially, 7H9 broths were inoculated (1 mL) with each of the isolates of *M. avium* (one isolate per broth). The cultures were incubated for 24–48 h to obtain turbidity equivalent to a McFarland 1.0 standard (3.0×10^8 CFU/mL). A separate BACTEC 12B vial (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA) was then inoculated (0.1 mL) with each of the isolates of *M. avium*. After incubation for 24 h at 37 °C, aliquots (0.1 mL) of those seed cultures (working suspension) with a growth index (GI) of 999 detected with the BACTEC 460 instrument were diluted 1 : 10 and 1 : 100 with BACTEC diluting fluid. Subsequently, 0.1 mL of the diluted suspensions of *M. avium* was used to inoculate each of the drug-containing BACTEC 12B vials and a drug-free control. Frozen vials containing each antimycobacterial agent were thawed, and 0.1 mL was injected into BACTEC 12B vials using a fixed-needle allergist syringe (Becton Dickinson). The final concentrations were: 2.0 mg and 8.0 mg of amikacin/L; 1.0 mg and 4.0 mg of ciprofloxacin/L; 2.0 mg and 32.0 mg of clarithromycin/L; and 0.12 mg and 2.0 mg of rifabutin/L. The vials were then incubated at 37 °C and tested with the BACTEC 460 instrument at approximately the same time each day. The minimum inhibitory concentration (MIC) was interpretable when the GI of the 1 : 100 control read 20 or more for three consecutive days while the GI of the undiluted control read 999. These requirements had to be met between days 4 and 8 of incubation for the test to be considered valid. The MIC was the lowest concentration of the antimycobacterial agent that inhibited 99% of the bacterial population. The final GI was less than 50.

Statistical analysis

A *t*-test was used to determine significant differences between the number of mycobacteria in the drug-free and drug-containing media. The alpha level was set at 0.05 before the experiments were started.

RESULTS

Determination of optimal incubation time for detection of *M. avium* after exposure to antimycobacterial agents by flow cytometry

M. avium isolate 933 was incubated in the presence of 0.01, 0.5, 2.0 or 8.0 mg/L of amikacin, ciprofloxacin, clarithromycin and rifabutin or in the absence of these antimycobacterial agents for 24 or 48 h before samples were analyzed by flow cytometry (Figure 1). The number of *M. avium* organisms detected in each culture containing 2.0 or 8.0 μg of amikacin, 0.5, 2.0 or 8.0 μg of ciprofloxacin or clarithromycin and 0.01, 0.5, 2.0 or 8.0 μg of rifabutin was significantly less ($P < 0.01$) than the number of organisms detected in the drug-free controls after a 24-h incubation. With a 48-h incubation, significantly fewer ($P < 0.01$) *M. avium* organisms were detected in all antimycobacterial-containing samples, except those containing 0.01 mg/L of amikacin, ciprofloxacin or clarithromycin. Similar results were obtained when these studies were repeated with nine additional isolates of *M. avium*. In subsequent studies we therefore routinely used a 24-h incubation to determine the susceptibilities or resistances of isolates of *M. avium* to antimycobacterial agents. Only in the unusual circumstance of a slowly growing isolate were results reported from samples incubated for 48 h.

Susceptibility of clinical isolates of *M. avium* to antimycobacterial agents

Eleven clinical isolates of *M. avium* with susceptibilities to amikacin, ciprofloxacin, clarithromycin and rifabutin previously established by the tuberculosis unit of the WSLH were tested for susceptibility to these four antimycobacterial agents with the 24-h flow cytometric technique. Figure 2 shows the flow cytometric susceptibility index values obtained with three isolates of *M. avium* each exposed to amikacin (isolates 175, 244 and 768), ciprofloxacin (isolates 141, 250 and 730), clarithromycin (isolates 141, 510 and 825) or rifabutin (isolates 244, 266 and 455). Isolate 175 was susceptible to 0.5 mg or more of amikacin/L, whereas isolate 244 was susceptible to 8.0 mg or more of amikacin/L (Figure 2a). Isolate 730 was resistant to all concentrations of ciprofloxacin. Isolate 141 was susceptible to 0.5 mg or more of clarithromycin/L (Figure 2b), while isolate 510 was susceptible only to 32.0 mg or more of clarithromycin/mL. By contrast, isolate 768 was resistant to all concentrations of amikacin tested. Isolates 141 and 250 were susceptible to 0.25 mg and 4.0 mg or more of ciprofloxacin/L, respectively (Figure 2c). Isolate 825 was resistant to all the concentrations of clarithromycin tested. Isolate 455 was resistant to all concentrations of rifabutin, while isolates 266 and 244 were susceptible to 0.05 mg and 2.0 mg or more of rifabutin/L, respectively (Figure 2d).

Comparison of susceptibility test results by the flow cytometric and BACTEC methods

Twenty isolates of *M. avium* were tested for susceptibility to amikacin, ciprofloxacin, clarithromycin and rifabutin by the flow cytometric and BACTEC methods (Table 1). Agreement (95%) between the methods was reached for 37 of the 39 tests performed on amikacin (2.0 mg/L), ciprofloxacin (1.0 mg/L), clarithromycin (2.0 mg/L) and rifabutin (0.12 mg/L). The two exceptions were isolates 933 and 045, which were resistant to 2.0 mg of amikacin/L or 1.0 mg of ciprofloxacin/L by flow cytometry, but susceptible to these concentrations of amikacin

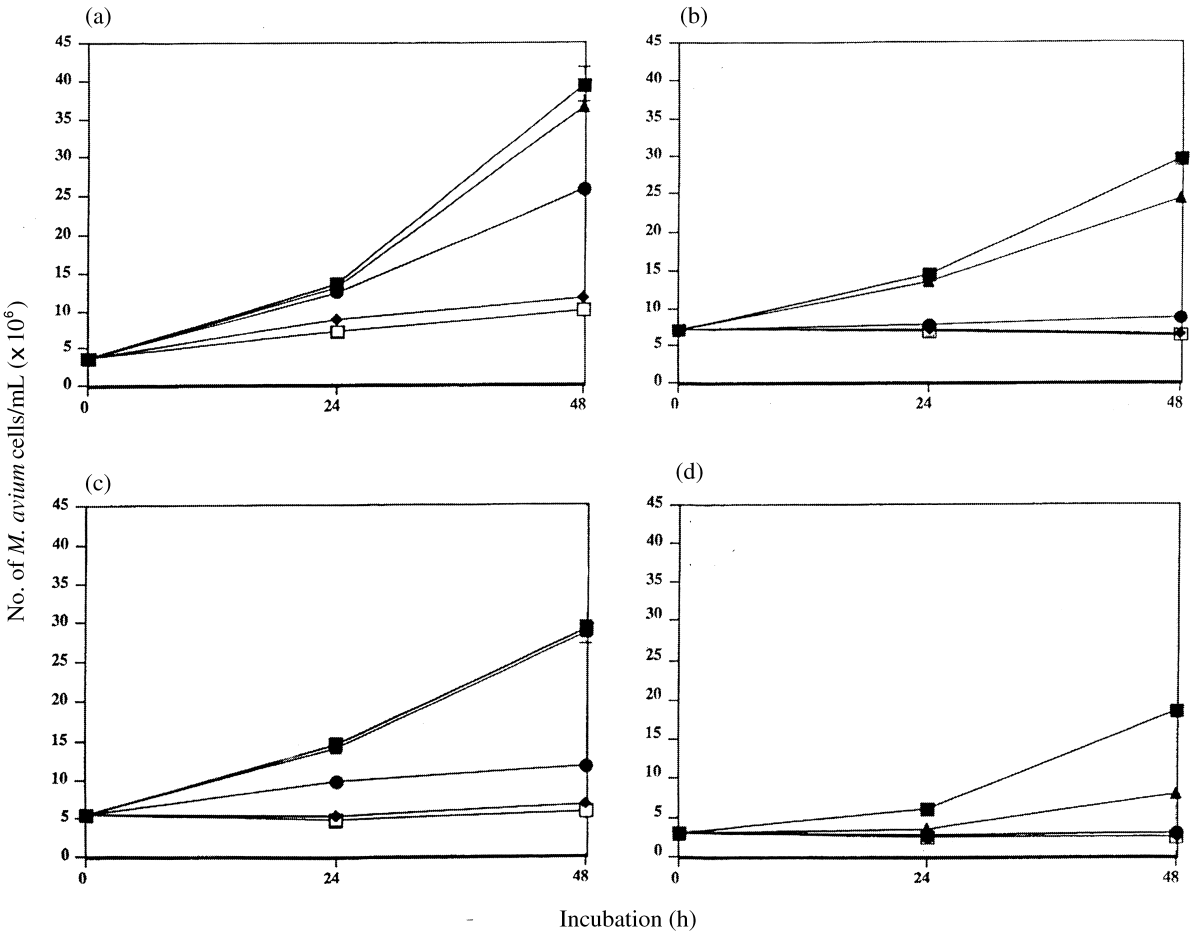

Figure 1 Number of *M. avium* cells (events)/mL detected by flow cytometry with or without (■) exposure to 0.01 (▲), 0.5 (●), 2.0 (◆) or 8.0 (□) mg/L of amikacin (a), clarithromycin (b), ciprofloxacin (c), or rifabutin (d) for 24 or 48 h. The experimental error was ±0.26, 0.24, 0.15 and 0.11 for amikacin (a), clarithromycin (b), ciprofloxacin (c), and rifabutin (d), respectively.

or ciprofloxacin by the BACTEC method. When higher concentrations of amikacin (8.0 mg/L), ciprofloxacin (4.0 mg/L), clarithromycin (32.0 mg/L) and rifabutin (2.0 mg/L) were tested by the flow cytometric and BACTEC methods, however, agreement was 100%. The overall agreement was 97% (76 of 78 tests).

Reproducibility

Table 2 shows the reproducibility of the flow cytometric susceptibility test. Four isolates (266, 045, 141, 569) with varying susceptibilities to amikacin, ciprofloxacin, clarithromycin or rifabutin were tested four times. Isolates 266, 045, 141 and 569 had susceptibility indices of 0.62 ± 0.02, 0.63 ± 0.03, 0.50 ± 0.02 and 0.75 ± 0.06 for amikacin (2.0 mg/L), ciprofloxacin (1.0 mg/L), clarithromycin (2.0 mg/L) and rifabutin (0.12 mg/L), respectively. Similar reproducibility was achieved with the higher concentrations of amikacin, ciprofloxacin, clarithromycin and rifabutin.

DISCUSSION

There is no standard method for performing susceptibility testing of isolates of MAC [3,13,15–18]. Conventional methods used for susceptibility testing of *M. tuberculosis* [23] are generally not recommended for testing MAC [1–3,11,13,15]. In fact, susceptibility testing of MAC is not recommended by the Centers for Disease Control and Prevention [18]. Despite these concerns, many clinical mycobacteriology laboratories continue to perform susceptibility testing for MAC, because

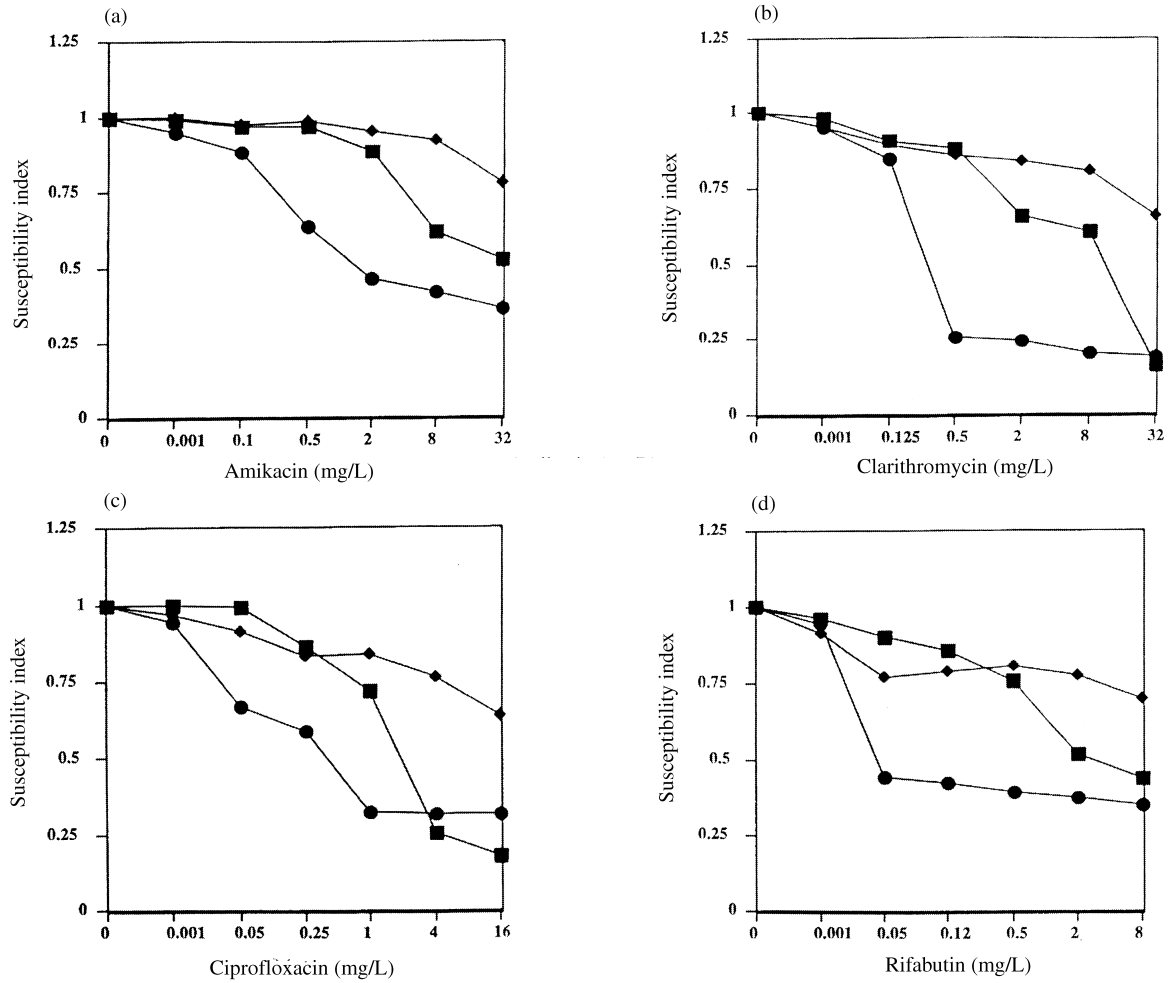

Figure 2 Susceptibility index values of different isolates of *M. avium* (◆ = 768, ■ = 244, ● = 175 for (a); ◆ = 825, ■ = 510, ● = 141 for (b); ◆ = 730, ■ = 250, ● = 141 for (c); and ◆ = 455, ■ = 244, ● = 266 for (d), with or without exposure to selected concentrations of amikacin (a), clarithromycin (b), ciprofloxacin (c), or rifabutin (d) for 24 h.

clinicians, especially those with extensive experience with mycobacterial disease, find susceptibility testing of MAC useful for guiding therapy. The most commonly used methods are the BACTEC TB [3,9,15,16,18] and quantitative minimal inhibitory or bactericidal concentration methods [1,19,24]. However, considerable efforts have been made to develop faster, more accurate, more reliable and biologically safe susceptibility testing methods for predicting clinical efficacy of antimycobacterial agents [1,16,18,25].

Our results show that susceptibility testing of *M. avium* can be accomplished rapidly by using a flow cytometer; test results are available within 24–48 h, compared to 4–8 days with the BACTEC method [3,9,15,16,18]. The assay depends on detection and enumeration of actively growing *M. avium* organisms in drug-free and antimycobacterial agent-containing media. In addition, the assay was safe. Mycobacteria were killed by exposure to paraformaldehyde before analysis with a flow cytometer.

Our flow cytometric test results of 20 clinical isolates of *M. avium* for susceptibility to amikacin, ciprofloxacin, clarithromycin and rifabutin agreed with the BACTEC method in 76 of the 78 (97%) tests performed. The only discrepancies (two tests) were *M. avium* isolates 933 and 045, which were resistant to 2.0 mg of amikacin/L and 1.0 mg of ciprofloxacin/L by the flow cytometric method, but susceptible by the BACTEC method. When higher concentrations of these antimycobacterial agents were tested, both isolates were susceptible to amikacin or ciprofloxacin by the flow cytometric and BAC-

Table 1 Results of susceptibility tests by the flow cytometric and BACTEC methods for isolates of *M. avium* exposed to amikacin, ciprofloxacin, clarithromycin or rifabutin.

	Results obtained by:			
	Flow cytometric method		**BACTEC method**	
Amikacin	2.0 mg/L	8.0 mg/L	2.0 mg/L	8.0 mg/L
028	S	S	S	S
045	R	S	R	S
141	R	S	R	S
175	S	S	S	S
244	R	S	R	S
266	S	S	S	S
316	R	R	R	R
569	R	S	R	S
768	R	R	R	R
923	R	R	R	R
933	R	S	S	S
Ciprofloxacin	1.0 mg/L	4.0 mg/L	1.0 mg/L	4.0 mg/L
045	R	S	S	S
141	S	S	S	S
244	S	S	S	S
250	R	S	R	S
569	R	R	R	R
730	R	R	R	R
768	R	S	R	S
871	R	R	R	R
933	S	S	S	S
Clarithromycin	2.0 mg/L	32.0 mg/L	2.0 mg/L	32.0 mg/L
077	S	S	S	S
141	S	S	S	S
250	R	R	R	R
510	R	S	R	S
730	R	S	R	S
768	R	R	R	R
776	S	S	S	S
825	R	R	R	R
933	S	S	S	S
Rifabutin	0.12 mg/L	2.0 mg/L	0.12 mg/L	2.0 mg/L
141	R	S	R	S
175	S	S	S	S
244	R	S	R	S
266	S	S	S	S
316	R	S	R	S
354	R	R	R	R
455	R	R	R	R
569	R	S	R	S
871	R	R	R	R
933	S	S	S	S

Isolates of *M. avium* were exposed to 2.0 or 8.0 mg of amikacin/L, 1.0 or 4.0 mg of ciprofloxacin/L, 2.0 or 32.0 mg of clarithromycin/L and 0.12 or 2.0 mg of rifabutin/L using the flow cytometric and BACTEC methods. Isolates marked 'S' for flow cytometry had susceptibility indexes of 0.65, 0.60, 0.55 and 0.55 or less after exposure to amikacin, ciprofloxacin, clarithromycin and rifabutin, respectively, for 24 h. The isolates marked 'S' for the BACTEC method showed less than 1% of growth detected in the drug-free control vials after 4–8 days of incubation. Isolates marked 'R' for both methods were considered to be resistant.

TEC methods. A likely explanation for the discrepancies lies in the cut-off value for the flow cytometric susceptibility index. We conservatively set the cut-off value at 0.65 and 0.60 for amikacin and ciprofloxacin, respectively. When the susceptibility index value was raised to 0.67 or 0.61, these isolates were categorized as susceptible to amikacin or ciprofloxacin.

Table 2 Reproducibility of the flow cytometric susceptibility test for four isolates of *M. avium* with varying susceptibilities and resistances to amikacin, ciprofloxacin, clarithromycin or rifabutin.

		Values for flow cytometric method		
Isolate	**Results of flow cytometric method**	**Antimycobacterial agent (concentration)**	**Mean susceptibility index**	**Standard deviation**
266		Control	1.00	0.00
	S	Amikacin (2.0 mg/L)	0.62	0.02
	S	Amikacin (8.0 mg/L)	0.54	0.03
045		Control	1.00	0.00
	R	Ciprofloxacin (1.0 mg/L)	0.63	0.03
	S	Ciprofloxacin (4.0 mg/L)	0.57	0.01
141		Control	1.00	0.00
	S	Clarithromycin (2.0 mg/L)	0.50	0.02
	S	Clarithromycin (32.0 mg/L)	0.42	0.04
569		Control	1.00	0.00
	R	Rifabutin (0.12 mg/L)	0.75	0.06
	S	Rifabutin (2.0 mg/L)	0.41	0.06

This minor adjustment in the susceptibility index resulted in complete agreement between the flow cytometric susceptibility test and BACTEC method. Although testing of more isolates might fine-tune the best-fit cut-off values, these values are unlikely to differ greatly from our selected values for amikacin (0.65), ciprofloxacin (0.60), clarithromycin (0.55) and rifabutin (0.55).

The flow cytometric susceptibility test required only 24 h after initiation of the testing procedure to obtain results. By contrast, the BACTEC method required 4–8 days of incubation before the susceptibility results were available [3,9,15,16,18]. Furthermore, approximately 15% of the results obtained by BACTEC required replication of the procedure. This further delayed reporting of results. Occasionally, slow-growing isolates of *M. avium* also delayed results of the flow cytometric susceptibility test. However, results were available after incubation for an additional 24 h after the initial flow cytometric analysis. Although we reported isolates 045 and 933 as resistant to 2.0 mg of amikacin/L and 1.0 mg of ciprofloxacin/L, respectively, these isolates were susceptible to these concentrations of amikacin or ciprofloxacin after incubation for 48 h. This is a major advantage of the flow cytometric susceptibility test procedure. The original suspensions of *M. avium* with or without antimycobacterial agents can be re-analyzed daily, if necessary. The BACTEC method, however, would require inoculation of a new set of 12B medium with and without antimycobacterial agents and incubation for 4–8 days before results become available.

Another major advantage of the flow cytometric susceptibility test is that it can generate a quantitative MIC. Multiple concentrations of each antimycobacterial agent can be tested with little additional expense or time. By contrast, the BACTEC method uses two points or critical concentrations to obtain results. The broad variation in the degree of susceptibility or resistance among isolates of *M. avium* is a valid argument against employing only critical concentrations for determination of results [15]. BACTEC can, of course, be performed with multiple concentrations of antimycobacterial agents, but this would increase the cost of the procedure. Finally, the large size of the inoculum used in the flow cytometric studies has a distinct advantage. Drug-resistant mutants have been found by daily re-analysis of the suspensions of *M. avium* with or without antimycobacterial agents. The larger the inoculum, the greater the chance of detecting resistant organisms. Expanded studies are still needed, however, to correlate the findings obtained by flow cytometry to the clinical outcome of *M. avium* disease in patients.

We showed previously that flow cytometry could also be used for susceptibility testing of *M. tuberculosis* [26–29] and other mycobacteria [21]. A major concern about flow cytometric testing of pulmonary pathogens has always been biosafety. It is possible that aerosolization of mycobacteria could occur during processing of the drug-free and drug-containing samples. Recently, we showed that treatment of the flow cytometric susceptibility suspensions with 1% paraformaldehyde eliminates the risk of infection to healthcare workers using the flow cytometer. No viable tubercle bacilli were recovered from the drug-containing or drug-free suspensions 40 min after treatment with paraformaldehyde. Accurate results could still be obtained, however, if the paraformaldehyde samples were analyzed 24 or 72 h after treatment. In this study, all samples containing isolates of *M. avium* were treated with parafor-

maldehyde, and no viable mycobacteria were recovered on 7H10 agar medium after treatment with paraformaldehyde and incubation for 3 weeks. Treatment of samples with paraformaldehyde therefore effectively eliminates concerns about the biosafety of the procedure.

We conclude that the advantages of speed, simplicity, reproducibility and safety offered by flow cytometry make it the method of choice for susceptibility testing of *M. avium* recovered from clinical infections.

ACKNOWLEDGMENTS

We thank Bio-Rad Laboratories in cooperation with the Gundersen Lutheran Medical Foundation, Inc., La Crosse, WI, for support.

We also thank Louise Kubista, Michelle Bussen, Donna Sweeney, Justin Shemanski, Terrence Kurzynski, Scott Kirk, Andrea Moore, Monica Remington and John Christopherson for excellent advice and assistance.

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